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Fifth day of October 2004

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PROVISIONAL SPECIFICATION

Fungal Host for Expression and Production of Recombinant Products

The invention is described in the following statement:

Technical Field

The present invention relates to fungal hosts, fungal vectors, and methods for producing recombinant products from fungal hosts.

Background Art

The ascomycete *Ophiostoma* spp. comprise common sap-staining fungi such as *O. piceae* that cause discoloration of wood. Some albino variants of *O. flocossum* have been used as biological control agents to prevent sap-staining (Held *et al.*, 2002. Holzforschung. Submitted). A fungus capable of invading wood via the parenchyma cells must secrete enzymes outside the growing mycelium in order to utilize the non-structural components of sapwood (eg. sugars, proteins and extractives). Secreted enzymes such as lipases (Brush *et al.*, Bio. Med. Chem. 7:2131-2138, 1999) and proteinases (Abraham and Breuil, Enzyme Microb. Technol. 18:133-140, 1996) from *Ophiostoma* have been studied in some detail. However, in addition to these studies, very little has been published about biochemical and genetic properties of *Ophiostoma*.

The present inventors have developed *Ophiostoma* as a host for expression of industrially important enzymes and biomolecules. To our knowledge, *Ophiostoma* would constitute a novel expression system, not described in this respect in the literature.

Disclosure of Invention

In a first aspect, the present invention provides an isolated *Ophiostoma* species capable of containing an expression vector and acting as a host for the expression of a recombinant product encoded by the expression vector.

Preferably, the expression vector contains a promoter functional in *Ophiostoma* and nucleic acid encoding selected endogenous or heterologous gene product(s).

Preferably, the isolated *Ophiostoma* species is *Ophiostoma floccosum*. More preferably, the *Ophiostoma floccosum* is selected from strains J2004, J2015, J2026, J2098 or J2188, as herein defined.

In a more preferred embodiment, the isolated *Ophiostoma* species is *Ophiostoma floccosum* strain J2026 or its descendant.

Preferably, the *Ophiostoma* species according to the present invention is characterised by:

(a) one nucleus per conidium/blastospore;

(b) conidia having mean spore size no less than 2-3 µm in diameter; and

(c) capable of secreting higher levels of a protein into culture medium when compared to the secretion of the parent strain.

The isolated *Ophiostoma* species has preferably undergone modification / selection to alter its ability to carry out or perform protein secretion.

It has been found by the present inventors that modifying / altering DNA by random mutagenesis creates a more adaptable *Ophiostoma* species to act as a host for gene expression.

Ophiostoma species according to the present invention differs from naturally occurring *Ophiostoma* species in its enhanced ability to excrete proteins into the cultivation medium.

In a second aspect, the present invention provides an expression vector suitable for use in *Ophiostoma* species, the vector comprising:

(a) a nucleic acid molecule defining a promoter derived from an *Ophiostoma* species or derived from some other source being capable of controlling the expression from the vector in an *Ophiostoma* species;

(b) one or more restriction enzyme sites for inserting a nucleic acid molecule encoding a recombinant molecule;

(c) a nucleic acid molecule encoding a selectable marker; and

(d) a nucleic acid molecule encoding a terminator sequence.

Preferably, the promoter is a *hex1* gene promoter or another strong promoter controlling the production of any homologous protein from an *Ophiostoma* species, preferably from *Ophiostoma floccosum*.

Preferably the restriction sites are selected from a number of unique restriction enzyme sites that are not present on the expression vector promoter, terminator and selection marker DNA fragments. More preferably, the restriction sites are for non-frequent cutting restriction enzymes that recognize at least six base pair restriction sites.

Preferably the encoded selectable marker provides antibiotic resistance, fluorescence, metabolic activity or complements a metabolic requirement. More preferably, the antibiotic resistance is hygromycin B or G418.

Preferably the nucleic acid molecule encoding a terminator sequence is selected from DNA encoding a highly secreted protein.

In a third aspect, the present invention provides a method for altering the characteristics of a fungal species, the method comprising introducing an expression vector according to the second aspect of the present invention into a fungal host.

Preferably, the introduction of the vector is by biolistic transformation.

Preferably, the fungal host is an *Ophiostoma* species according to the second aspect of the present invention.

In a fourth aspect, the present invention provides a recombinant *Ophiostoma* species capable of expressing a product selected from protein, peptide, enzyme, or any product with potential in an industrially relevant application.

In a fifth aspect, the present invention provides use of a modified fungal species in an industrial process such as pulping, bleaching, recombinant protein production, and any other suitable process utilizing microbial production or action.

An advantage of using a fungal species (*Ophiostoma*) instead of bacteria or yeasts is that *Ophiostoma* is naturally living on wood for *in situ* delivery of selected gene products.

The present invention is particularly suitable for developing modified fungal species which are capable of excreting extracellular components such as enzymes that can be applied to processes either as liquid form from submerged fermentation or delivered *in situ* by the fungus growing on a particular material in solid state.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of the present invention.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps,

but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following drawings and examples.

Brief Description of the Drawings

Figure 1 shows the gene expression cassettes for foreign protein expression in *Ophiostoma*. All DNA fragments in a cassette have been constructed using the plasmid pUC19 as the carrier for replication in *E. coli*.

A. Basic expression vector with gene of interest fused to the signal sequence.

B. Expression vector to test the different gene promoter strength and secretion signal sequence function using the fluorescent *dsRed1-E5* (Ds-Red) as a reporter gene.

C. Expression vector with the gene of interest fused to the pre-pro-fragment (Prp) for assistance in folding, if required. Highlighted features include any gene promoter represented by P, any secretion signal sequence, SS, proteolytic Kex2-like cleavage site, RQ, multiple cloning site, MCS, selectable marker gene, M (eg. fluorescent or antibiotic), and the appropriate gene terminator by T. Positions for unique restriction enzyme sites are shown by X.

Figure 2. SDS-PAGE separation of secreted proteins by *Ophiostoma* J2026 culture grown in a minimal medium containing mineral salts, soy-hydrolysate and starch (pH 6.5). The lanes 1-4 mark culture supernatants from days 1-4. M, molecular weight marker. Circled protein bands have been cut out and N-terminally sequenced. The approximately 33 kDa band (black circle) has been identified as serine protease (AYTTQTGAPW). The other abundant proteins, the approximately 70 kDa protein (purple circle) with an N-terminal sequence of ATFPKASVTV and the ~110 kDa protein (red circle) with an N-terminal sequence of AVG(G)V(R) have not been identified.

Figure 3. SDS-PAGE separation of the proteins secreted by *Ophiostoma* J2026 and selected first round mutants. M, molecular weight markers. Lane 1, strain J2026; lane 2, strain J2027; lanes 3-9, strains J2027.1-J2027.7, respectively.

Mode(s) for Carrying Out the Invention

In order to obtain the present invention, the first step was to develop an effective transformation system for *Ophiostoma*, which could serve one or more of three

purposes: (i) introduction of novel properties in the fungus for biological control; (ii) introduction of novel enzyme activities such as ligninases in the fungus for biopulping purposes, and (iii) development of *Ophiostoma* as a novel system for the expression of a variety of industrially important gene products, either homologous or heterologous (enzymes, biopharmaceuticals, etc).

Transformation of *Ophiostoma* protoplasts with an *Aspergillus* plasmid has been described earlier (Wang *et al.* Mycol. Res. 103:77-80, 1999). The present inventors believe that the use of protoplasts is undesirable, first because of regeneration difficulties, and secondly, because of the instability originating from the presence of more than one nucleus per cell compartment in many *Ophiostoma* species. The approach of the present invention involved development of a complete expression system including design and construction of a series of vectors for effective gene expression, transformation of *Ophiostoma* using particle bombardment (Hazell *et al.* Lett. Appl. Microbiol. 30:282-286, 2000) and screening of mutant strains for improved secretion properties to be used as expression hosts.

Basic requirements for a potential expression host strain, before commencing with biolistic transformation, are: (i) preferably one nucleus per blastospore/conidium; (ii) occurrence of (blasto)spores with a minimum length and diameter of 2-3 µm; and (iii) sensitivity to a potent antibiotic (eg. hygromycin B) at a manageable level (eg. 100 to 200 U/ml). Experiments on *Ophiostoma floccosum* strains J2004, J2015, J2026, J2098 and J2188 have shown that the mutagenized-derived strains J2027 and 2027.1-2027.7 from J2026 display necessary features and therefore have been chosen as the preferred hosts for further development.

MATERIALS AND METHODS

Construction of expression vectors

An expression vector preferably requires a strong promoter for gene expression, a secretion signal for targeting of the gene product outside the fungal mycelium, and a suitable marker gene for transformant selection.

The present inventors have identified highly produced intracellular and secreted proteins using 1D and 2D gel electrophoresis and N-terminal amino acid sequencing. Promoters of the genes encoding such proteins are good candidates for driving efficient expression of genes linked to them. In the first phase, the homologous promoter *of hex1*

conferring expression of one of the major proteins in the fungal cell envelope (Lim *et al.*, Proteomics 1:899-910, 2001) and the promoter of a serine protease gene were isolated.

The isolation of the *hex1* gene from the filamentous fungus *Trichoderma reesei* has been achieved by some of the present inventors. A similar approach described below was taken to isolate the homologous *hex1* gene and sufficient promoter and terminator DNA fragments from the fungus *Ophiostoma floccosum*. The *hex1* gene promoter from *O. floccosum* (strain J2026) was isolated using the Genomic Walking PCR method (Morris *et al.*, Appl. Environ. Microbiol. 64:1759-1765,1996). Initially, a small portion of the *hex1* coding region was amplified using degenerate primers. The fragment was sequenced to confirm that it encoded the HEX1 protein sequences. This was followed by the design of new upstream and downstream walking primers which were used together with nested linker primers to obtain the rest of the *hex1* gene coding DNA, as well as sufficient promoter and terminator region fragments.

More specifically, degenerate primers for the *hex1* gene (hex1fwd.pr: 5'- ACA TCT TCC AAA ATG GGN TAY TAY GA -3' and hex1rev.pr: 5'- ACG GGG CCG CAC ATN GTY TGN AC –3') were designed based on nucleotide translation of conserved amino acid sequences from an alignment of HEX1 peptide sequences (Lim *et al.*, 2001. Proteomics 1:899-910; Jedd and Chua, 2000. Nature Cell Biology 2:226-231). In addition to the *Ophiostoma hex1* protein encoding sequence, an approximately 2 kb fragment of the 5' region (promoter) and 1.5–2 kb of the 3' region (terminator) were isolated. The promoter and terminator fragments were then used in the design of the *hex1* based expression vector.

A serine protease was identified as an effectively secreted protein in *Ophiostoma* strain J2026 grown in liquid medium (see Figure 2). A dominant protein band of approximately 33 kDa in size was cut out and N-terminally sequenced. This protein was identified as a serine protease. The corresponding gene *(osp1)* and its flanking sequences were isolated using GWPCR. Concensus primers (protfwd.pr, 5'-GGGGTCTSGCYCGTRTCTCCCA-3' and protrev.pr 5'-GCGGTGGWGCTGCCRATCCA-3') were designed based on conserved amino acid sequences from alignment of protease peptide sequences (Hoffman and Breuil, Curr. Genet. 41:168-175, 2002) and used to amplify most of the *osp1* gene. In addition to the *Ophiostoma* serine protease encoding gene sequence, an approximately 2 kb fragment of the 5' region (promoter) and 2 kb of the 3' region (terminator) were isolated. The promoter and terminator fragments were then used in the design of the *osp1* promoter based expression vector.

Similarly, the genomic walking PCR method can also be used to isolate, for example, lipase, amylase or glucoamylase gene promoters and their secretion signal sequences.

In addition to promoter and terminator sequences, the expression cassette may contain, for example, the *T. reesei* CBHI secretion signal sequence or a signal sequence from the secreted host serine protease, lipase, amylase or glucoamylase enzyme, as well as a multiple cloning site for inserting any foreign DNA.

Tests carried out to determine promoter strength as well as secretion signal sequence function involved the use of a gene encoding a fluorescent foreign protein such as *dsRed1-E5* (Terskikh *et al.*, Science, 290:1585-1588, 2000) fused to the different signal sequences and promoters. The best promoter-secretion signal sequence combinations were then used in future protein expression work using the high secreting *O. floccosum* host strain(s) described below.

Screening for high secretors

Secretion capacity of filamentous fungi can be improved by random mutagenesis and screening for enhanced and favourable characteristics. This procedure involves the treatment of fungal spores with physical (UV) or chemical mutagens, followed by screening for better secretors using a plate assay.

A 20 ml spore suspension of *Ophiostoma floccosum* (strain J2026) was placed on a 20 cm glass Petri dish at a concentration of 5×10^8 spores/ml and irradiated at 254 nm UV-light for a period of 0 to 8 min. One ml samples were removed at one minute intervals, diluted in 0.9% NaCl, 0.01% Tween-80 solution and plated on Potato Dextrose Agar + 0.1% Triton X100-plates for counting. Colonies from the 5-50% survival level were picked and patched onto minimal agar plates (Penttilä *et al.*, Gene 61: 155-164, 1987) supplemented with soy hydrolysate (1.5 % w/v) and insoluble starch (3% w/v) for the screening of starch-degrading enzymes (e.g. amylase, glucoamylase). The presence of clearing halos around the colonies indicates starch degrading activity. Relative size of the hydrolysis halo around a mutant colony gives an indication of enzyme secretion when compared to the nonmutagenized parent. It will be appreciated that other screening means known to the art can also be used.

Colonies showing considerably larger halos when compared to the parent strain were grown in shake cultures in a medium promoting production of a wide variety of hydrolytic enzymes.

The basal liquid medium for general protein production contained 15 g KH_2PO_4,

5 g $(NH_4)_2SO_4$, 10 ml 100 x trace elements (100 mg $FeSO_4 \times 7H_2O$, 20 mg $MnSO_4 \times H_2O$, 20 mg $ZnSO_4 \times 7H_2O$, 40 mg $CoSO_4 \times 7H_2O$ in 200 ml MQ water) and MQ water to a final volume of 1 litre. The pH was adjusted to 6.5. The basal medium was divided into 50 ml portions in 250 ml conical flasks and soybean flour (consisting of 52% protein, 1% fat) was added to each flask to a final concentration of 1.5% (w/v) for induction of e.g. lipase and protease production. The carbon source used for amylase induction was 3% (w/v) filter sterilized potato soluble starch (Sigma Chemicals). Each flask was inoculated with 10^8 spores and cultivated for 5 days at 28 °C and 250 rpm.

Enzymes secreted into the cultivation medium were assayed using previously published methods. Protease activity was monitored as in (Lovrien et al. J. Appl. Biochem. 7:258-272, 1985) and the method for amylase and glucoamylase activity has been described in Bailey and Nevalainen (Enzyme Microb Technol. 153-157, 1981). SDS-PAGE analysis of secreted proteins from the culture supernatants was carried out in 12.0 % (w/v) and 4-20 % (w/v) gradient acrylamide gels according to Laemmli (Nature 227, 680-685, 1970).

Biolistic transformation

Transformation was carried out using particle bombardment of fungal spores as outlined in Hazell *et al.* (Lett. Appl. Microbiol. 30:282-286, 2000) using a hepta-adaptor device that allows delivery of DNA into the conidia from seven barrels, thereby increasing the transformation efficiency (Te'o *et al.*, J. Microbiol. Methods, 51:393-399, 2002). Seven portions of 7 to 14 days old spores ($1 \times 10^7 - 1 \times 10^8$ in 0.9 % NaCl, 0.01% Tween 80 solution) were plated appropriately on PDA plates for hygromycin selection or MM plates containing acetamide as the sole nitrogen source for the selection of transformants (Penttliä *et al.*,1987) to align with the seven barrels of the Hepta Adaptor, and left to dry. Precipitation of circular and linear DNA onto tungsten particles (0.7 µm mean diameter, Bio-Rad) was carried out as described in Hazell *et al.*, (2000) with some minor modifications. For example, 100 µl of tungsten beads were used instead of 50 µl per DNA sample and accordingly, the amount of 2.5 M $CaCl_2$ and 0.1 M Spermidine were increased to 100 and 40 µl from 50 and 20 µl, respectively during the precipitation step. Following precipitation, the mixture of tungsten particles coated with DNA were resuspended in 80 µl of 100 % Ethanol, of which 10 µl (x 7) samples were removed and used for bombardment as described below. DNA concentrations of 1000 ng (x 7) were used in the experiments.

For bombardment, 10 µl of DNA-coated tungsten particles were loaded onto seven macrocarrier disks sitting in the seven slots of the Hepta Adaptor holder and left to dry. A single rupture disk of 1350 *psi* strength was placed on the top to temporarily block the helium gas from entering the seven barrels of the Hepta Adaptor instrument. When dried, the Hepta Adaptor holder containing DNA-coated tungsten beads was placed inside the PDS-1000/He system chamber directly below the Hepta Adaptor device. PDA plates containing conidia for bombardment were placed at a target distance of 3 cm.

Following bombardment, PDA plates were incubated at 28 °C for 4-6 hrs before overlaying them with 10 ml of PDA containing hygromycin B to a final concentration of 150 U/ml or 90 µg/ml of G418. *Ophiostoma* transformants capable of using acetamide were identified based on growth of colonies on the bombarded MM acetamide plates after incubation at 28 °C.

Sorting of transformed conidia using flow-cytometry

Flow cytometric sorting was trialled to directly capture transfomants expressing the foreign DsRED protein. Bombardment of spores was carried out as discussed above. Before sorting, the promoter used in a particular vector was induced. For example, soybean flour, olive oil and starch were used to induce protease, lipase and amylase promoter vectors respectively. Spores bombarded with the *hex1* promoter vector were plated directly onto PDA plates.

Following bombardment, spores were incubated at 18 - 20°C with sufficient time for promoter induction to occur before harvested, purified and first checked under a fluorescent microscope before sorting the highly fluorescent spores using the flow cytometer onto a membrane, which can be removed and plated onto selective plates.

RESULTS

Expression vectors

Preferred expression vectors are shown in Figure 1. Vectors described have the foreign gene fused to the appropriate signal sequence under the *Ophiostoma hex1/protease/lipase/amylase* or another highly expressed gene promoter. Figure 1 shows preferred gene expression cassettes for foreign protein expression in *Ophiostoma*. All DNA fragments in a cassette have been constructed using the plasmid pUC19 as the carrier for replication in *E. coli*. Figure 1A is a representation of basic expression vector with gene of interest fused to the signal sequence. Figure 1B is a

representation of an expression vector to test the different gene promoter strength and secretion signal sequence function using the fluorescent *dsRed1-E5* (Ds-Red) as a reporter. The *dsRed1-E5* protein is heterologous to *Ophiostoma* thus also providing and example of expression of a foreign protein in the *Ophiostoma* system. Figure 1C is a representation of an expression vector with the gene of interest fused to the pre-pro-fragment (Prp) for assisted folding, if required. Highlighted features include any gene promoter represented by P, any secretion signal sequence, SS, proteolytic Kex2-like cleavage site, RQ, multiple cloning site, MCS, selectable marker gene, M (eg. *amdS* or antibiotic), and the appropriate gene terminator by T. Positions for unique restriction enzyme sites are shown by X.

Identification and improvement of *Ophiostoma* strains suitable as expression hosts by SDS-PAGE analysis of secreted proteins

SDS-PAGE of secreted proteins was initially applied to recognize good protein secretors suitable for the development of expression hosts and to identify the most highly secreted proteins to isolate strong gene promoters driving their synthesis (discussed above). In order to profile the proteins secreted by *Ophiostoma* strains J2026, J2027 and J2027.1-J2027.7, culture supernatants were analysed on a 12% SDS-PAGE gel and stained with Coomassie stain (Figures 2 and 3).

In order to develop the strains further, screening for strains with increased protein secretion and/or modified enzyme profile after UV mutagenesis was carried out. Plate assays were used for the screening of mutants, for example, with improved starch-degrading activity.

With strain J2026, nine mutant colonies with potentially increased starch degrading activity were picked from the screening plates and grown in liquid culture as described in Materials and Methods. Glucoamylase and protease activity as well as total secreted protein were assayed from the culture supernatants (Table 1).

Table 1, Glucoamylase (GA) and protease activity of first round UV mutants of *Ophiostoma* 2026.

Strain	GA nkat/ml	Protease Units*	Total protein (mg/ml)
J2026 (parent)	10.3	4.43	2.28
J2027	36.83	0.25	2.62
J2027.1	18.4	13.25	2.36
J2027.2	18.23	13.03	2.13
J2027.3	13.35	4.97	2.20
J2027.4	11.91	7.12	2.87
J2027.5	10.48	0.48	2.00
J2027.6	9.99	13.41	2.05
J2027.7	9.53	5.81	2.46
J2027.8	7.41	2.86	2.69

* (as in Lovrien et al. 1985)

The mutant J2027 showed about three fold increase in the glucoamylase activity and 18 fold decrease in the protease activity when compared to the nonmutagenised parent J2026. In addition, secretion of the total protein was increased by 14 % in J2027. In the mutant J2027.4, the glucoamylase and protease activities were slightly increased when compared to the parent J2026, however, the total secreted protein was increased by 25 %. These examples show that total protein secretion and protein profiles of *Ophiostoma* can be modified by random mutagenesis. Low protease producing strains are of special interest as expression host for foreign gene products.

Figure 3 shows the proteins secreted by the mutants in Table 1, separated by SDS-PAGE highlighting the different protein profiles.

Transformation of *Ophiostoma* by biolistic bombardment

The average transformation efficiency of *Ophiostoma* was 4 transformants per microgram of DNA using 500 ng of DNA (*hygB* marker), 1350 *psi* and 3 cm target distance. Following bombardment, the transformants were tested on PDA containing

150 units per ml of hygromycin B and 90 µg per ml of G418 or identified by colony growth on minimal medium agar plates containing acetamide as the sole nitrogen source.

Sorting by fluorescence

The resultant *Ophiostoma* transformants can be sorted by flow cytometry based on fluorescence of the DsRED marker protein which can be excited at wavelenths of 483 nm (when the protein is first synthesized and will appear green in colour) and 558 nm (as the DsRED protein matures and will appear red). It will be appreciated that other screening means known to the art can also be used.

SUMMARY

We have devised an *Ophiostoma* system for efficient expression of selected gene products. The system comprises promoter(s) functional in *Ophiostoma,* suitable transformation markers and transformation of a suitable *Ophiostoma* strain by biolistic bombardment. We have shown that the amount of secreted protein of a fungal strain can be increased and protein profiles modified following UV-light mutagenesis. Mutagenesis also allows for the production of host strains with different background enzyme profiles. Enhanced secreting and low protease mutant strain(s) can be used as expression hosts in the production of industrially important molecules or proteins. The transformed mutant strain can be subjected to additional rounds of UV-light mutagenesis to further improve desired characteristics.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this twenty fourth day of September 2003

Macquarie University

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PATENT & TRADE MARKS ATTORNEYS

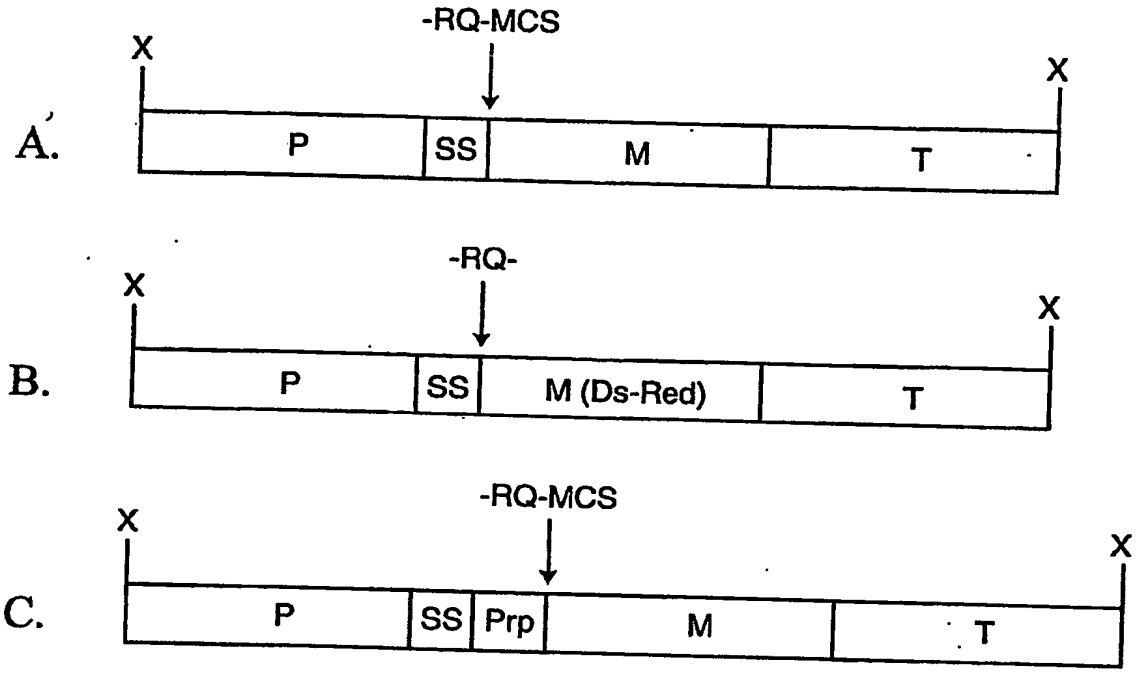

Figure 1

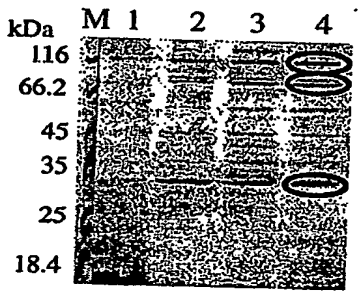

Figure 2

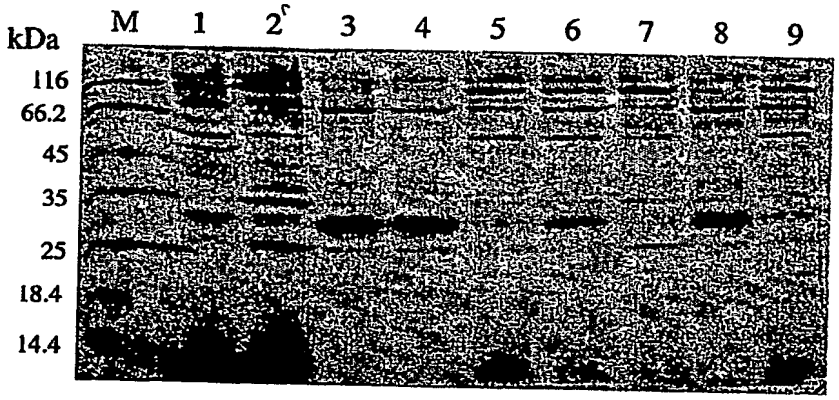

Figure 3